Pergamon

0022-1910(95)00038-0

J. Insect Physiol. Vol. 41, No. 10, pp. 857–867, 1995
Elsevier Science Ltd. Printed in Great Britain

Olfaction in a Hemimetabolous Insect: Antennal-specific Protein in Adult *Lygus lineolaris* (Heteroptera: Miridae)

JOSEPH C. DICKENS,*§ FRANKLIN E. CALLAHAN,† WILLIAM P. WERGIN,‡ ERIC F. ERBE‡

Received 16 December 1994; revised 27 February 1995

Antennal sensilla, electrophysiological responses to an insect and a plant odorant, and polypeptide profiles were investigated in fifth instar nymphs and adults of the tarnished plant bug, *Lygus lineolaris*. Sensilla with surface pits characteristic of porous olfactory sensilla develop on the second and third antennal segments during the final molt from fifth instar nymph to adult. Concurrent with development of these sensilla in the adult, neural responses to a component of green odor (1-hexanol) and an insect-produced volatile [(*E*)-2-hexenyl butyrate] increase dramatically. Antennal extirpation experiments indicated that sensilla responsive to these odorants are housed principally on the second and third antennal segments. A protein with a molecular weight of 17,000 present in the soluble fraction of adult antennae was absent in nymphs. Localization of this protein to the antenna, the coincidence of its expression with development of olfactory sensilla and its molecular weight were characteristic of odorant-binding proteins in moths. However, antisera raised against pheromone-binding protein of the gypsy moth, *Lymantria dispar*, did not react with the *Lygus* protein. The N-terminal sequence for our antennal-specific protein was determined, and showed no significant homology with other known insect protein sequences. This lack of homology with other insect proteins including odorant binding proteins indicates that if it is an odorant binding protein as we suspect, it is either widely divergent or independently derived. This is the first report of an antennal-specific protein in a hemimetabolous insect and the only report of such a protein in an insect other than moths or *Drosophila melanogaster*.

Olfaction Antennal-specific protein *Lygus lineolaris* Heteroptera Electrophysiology Development Morphology Sensilla Molecular biology

INTRODUCTION

The tarnished plant bug, *Lygus lineolaris* (Heteroptera: Miridae), is a hemimetabolous insect which undergoes five nymphal instars prior to molting to the adult. It is a pest of a number of crops including cotton and soybeans in the United States and Canada, and current control measures include early season insecticide treatments which are environmentally undesirable and may trigger outbreaks of other pests by concurrently destroying their natural enemies (Snodgrass *et al.*, 1984). *L. lineolaris* females attract conspecific males in both field experiments (Scales, 1968) and laboratory behavioral studies (Graham, 1987). Antennal extirpation experiments in the closely related species, *L. hesperus*, demonstrated the importance of the second and third antennal segments in the response of males to a female attractant in laboratory behavioral experiments (Graham, 1988).

Detection of volatile chemical messages by insects is accomplished by olfactory neurons housed within cuticular sensilla mostly on the antennae. According to current concepts derived mostly from studies of olfactory systems in moths, odorous molecules that are adsorbed in the surface layer of the antennal cuticle diffuse to pores in the sensilla. Movement to putative receptor sites on dendrites of olfactory receptor neurons may be along pore tubules extending across the receptor lymph (Kaissling, 1974) and/or, more likely, aboard odorant binding proteins (OBPs) located in the receptor lymph (Kaissling, 1986; Vogt and Riddiford, 1986). Interaction with a membrane-bound receptor leads to a change in membrane conductance thus producing a receptor potential. The molecule is then removed from the

*United States Department of Agriculture, Agricultural Research Service, Boll Weevil Research Unit, Mississippi State, MS 39762, U.S.A.

†United States Department of Agriculture, Agricultural Research Service, Cotton Host Plant Resistance Research Unit, Mississippi State, MS 39762, U.S.A.

‡United States Department of Agriculture, Agricultural Research Service, Electron Microscopy Laboratory, Beltsville, MD 20705, U.S.A.

§To whom all correspondence should be addressed.

membrane receptor by binding to an OBP (Kaissling, 1986) or is enzymatically degraded (Vogt *et al.*, 1985). The receptor potential spreads to an impulse generator where action potentials are generated.

In contrast with the amount of work reported on moths, few studies deal with development and molecular biology of olfaction, and the neural responses from sensilla of insects with incomplete metamorphosis. Schafer and Sanchez investigated olfactory development in cockroaches of the genus *Periplaneta* and showed that development of olfactory sensilla (Schafer and Sanchez, 1976a), sexual behavior (Schafer, 1977a) and neural responses to a sex attractant (Schafer, 1977b) were regulated by juvenile hormone. Levinson *et al.* (1974) showed that both final instar nymphal and adult bedbugs responded to an alarm pheromone with receptors on the terminal segment of the antenna. Electroantennograms recorded from adult milkweed bugs, *Oncopeltus fasciatus*, showed the antennae to have receptors for milkweed extracts (Pantle and Feir, 1976), and correlated with an earlier study in which antennectomy reduced the ability of the bugs to locate milkweed seeds (Feir and Beck, 1963).

We report here results of studies which reveal the presence of chemosensitive sensilla, electrophysiological responses to specific odors, and expression of an antennal-specific protein in the adult *L. lineolaris* that are not present in the final stage nymph.

MATERIALS AND METHODS

Insects

Fifth instar nymphs and adults of *L. lineolaris* were obtained from a laboratory colony annually infused with feral insects maintained at the USDA, ARS, Southern Insect Management Laboratory, Stoneville, MS (Snodgrass and McWilliams, 1992). Upon receiving insects at our laboratory, adults and fifth instar nymphs with abdominal sexual characters were sexed and separated into groups of three in plastic containers with paper tops. These insects were fed broccoli and held at 25°C and 14 h of light and 10 h of darkness until use, generally within 2 or 3 days.

Morphology

An Oxford CT 1500 Cryotrans System was mounted on a Hitachi S-4100 field emission scanning electron microscope (SEM) to perform low temperature manipulations and observations. Insect material (whole insects or excised head and antennae) was placed on the surface of a methyl cellulose solution (Tissue TekTM) that filled holes drilled into an Oxford specimen carrier. The carrier containing the specimens was then rapidly plunge-frozen in liquid nitrogen. A cryo transfer rod was attached to the carrier. The Oxford nitrogen slush chamber was evacuated, and the assembly was withdrawn into a cryo-transfer arm for transfer to the cold stage of the cryo-preparation chamber. The temperature of the stage was raised to −90°C for 8 min to remove water from the surface. The specimens were then sputter coated for 2 min with platinum prior to transfer to the cold stage of the Hitachi S-4100 SEM.

Accelerating voltages of 10 kV were used to observe and record images onto Polaroid Type 55 P/N film. To facilitate our analyses of nymphal and adult antennae, stereo pairs were recorded for many observational fields. To obtain stereo pairs, the first image was recorded, the stage was tilted 5–10 degrees, the specimen was recentered and a second image was recorded. Together these two images contained the parallax information necessary for stereo viewing.

Electrophysiology

Electroantennograms (EAGs) were recorded from adult males and fifth instar nymphs with male sexual characters using a modification of an earlier technique (Schneider, 1957; Dickens *et al.*, 1993). Antennae were excised near the middle of the first antennal segment. EAGs were recorded from: (1) adult males and nymphs in which the distal tip of the terminal antennal segment had been clipped; (2) adult males in which the terminal segment was removed near its junction with the third antennal segment; and (3) adult males in which the terminal segment and the penultimate segment were removed near the junction with the second antennal segment. An excised antenna was fixed between two glass capillary electrodes filled with either 0.1 M KCl or *Drosophila* Ringer. Ag–AgCl wires in the glass capillaries connected the preparation to the recording instruments: a Grass P-16 preamplifier, a Tektronix 5111A analog storage oscilloscope and a Houston Instruments stripchart recorder. Maximal depolarization of the EAG during the stimulation period was used as a measure of activity of the odorous stimuli. Three replicates were recorded for each experimental condition. For two insects, after recordings were made from one antenna with the distal segment removed, a second set of recordings were obtained from the contralateral antenna with both the distal and the penultimate segments removed. This allowed for direct comparisons in the same insect.

Odorous stimuli consisted of a component of green odor, 1-hexanol, which elicits EAGs from many insects (Visser, 1986), and (*E*)-2-hexenyl butyrate, a compound in greater abundance in *L. lineolaris* females than males (Gueldner and Parrott, 1978; Aldrich *et al.*, 1988). After dilution in nanograde hexane, 50 μg of an experimental odorant was placed on a piece of Whatman #1 filter paper (8 × 18 mm) as a 5 μl aliquot of a 10 μg/μl dilution. The filter paper was inserted into a glass tube (80 mm long × 5 mm i.d.) and oriented toward the preparation with the outlet 1 cm from the antenna. The hexane was allowed to evaporate for 15 s prior to use of the odor cartridge. Each stimulation lasted 1 s. The interstimulus time interval was 2–3 min. Five μl of the hexane solvent served as a control after evaporating for 15 s from the filter paper.

Polyacrylamide gel electrophoresis (PAGE)

Whole antennae were excised from the insects, collected in micro-tissue grinders (WheatonTM 357848) and stored at −70°C until use within a few days. Tissue samples consisted of 10–30 antennae which were collected from fifth instar nymphs with male or female sexual characters, and antennae from adults. Tissues to be analyzed by one-dimensional PAGE were homogenized on ice following addition of 30 μl of non-SDS–PAGE sample buffer (50 mM Tris–HCl, pH = 6.8; 10% glycerol). Homogenates were centrifuged (4°C) for 2 min at 12,000 ***g***. The resulting supernatants (soluble protein fractions) were collected and stored at −70°C. Soluble protein fractions were either used directly for native-PAGE, or aliquots were removed and mixed with an equal volume of 2 × SDS (denaturing)–PAGE sample buffer (125 mM Tris–HCl, pH = 6.8; 20% glycerol, 4% SDS, 10% mercaptoethanol) for analysis by SDS–PAGE. The SDS-containing samples were denatured by heating at 95°C for 5 min prior to electrophoresis. Such SDS-denatured samples were also used for two-dimensional PAGE analysis as described below. Samples for two-dimensional PAGE were spiked with 40 ng of tropomyosin (MW = 32.7 kDa, pI = 5.2) which served as an internal standard. Relative protein concentrations in the various samples were estimated from 1 μl aliquots (Marder *et al.*, 1986) and then equalized prior to electrophoresis by dilution with the appropriate buffer. Typically, the protein concentrations of the soluble protein fractions described above were about 0.1 mg/ml.

One-dimensional PAGE was as described by Laemmli (1970) using a mini-gel apparatus (Bio-RadTM). Native gels consisted of 4.5 and 12.5% acrylamide in the stacking and resolving gels, respectively. SDS was omitted from the Laemmli gel and running buffers. Electrophoresis was at 4°C with a constant current of 10 mA for 1 h followed by 15 mA (maximum voltage 200 V) until bromophenol blue tracking dye added to blank sample buffer lanes reached the bottom of the gel (about 4 h). SDS-gels consisted of 4.5 and 15% acrylamide in the stacking and resolving gels, respectively. SDS (0.1%) was present in the Laemmli gel and running buffers. Electrophoresis was at room temperature with constant 50 V for 1 h followed by 150 V until the tracking dye reached the bottom of the gel. Protein standards (Bio-RadTM high and low molecular weight standards) used for one-dimensional SDS gels were: (1) myosin = 200,000; (2) rabbit muscle phophorylase b = 97,400; (3) bovine serum albumin = 66,200; (4) hen egg white ovalbumin = 42,699; (5) bovine carbonic anhydrase = 31,000; (6) soybean trypsin inhibitor = 21,500; and (7) hen egg white lysozyme = 14,400. Immunoblots of one-dimensional gels were done essentially as described by Vogt *et al.* (1989) with the exceptions that blocking time was reduced to 4 h and the secondary antibody–conjugate complex used to visualize binding of the primary antibody (anti-PBP2 of *Lymantria dispar*) was anti-rabbit IgG alkaline phosphatase (PromegaTM).

Two-dimensional PAGE was performed according to the method of O'Farrell (1975) by Kendrick Labs Inc., Madison, WI. Isoelectric focusing in the first dimension was in glass tubes (2.0 mm i.d.) using 2.0% Resolytes pH 4–8 ampholines (Hoefer Scientific Instruments) for 9600 V-h. The pH gradient for this set of ampholines was confirmed by measurements with a surface pH electrode. Following electrophoresis in the first dimension, the tube gel was equilibrated for 10 min in 10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 62.5 mM Tris–HCl, pH = 6.8. The tube gel was then sealed to an SDS–PAGE gel consisting of a 4 and 12.5% acrylamide stacking and resolving gel, respectively, and run at room temperature at 4 mA constant current for 4–5 h. Protein standards (Sigma Chemical Co., St Louis, MO) used for the second dimension (SDS–PAGE) of the two-dimensional gels were: (1) myosin = 220,000; (2) phosphorylase A = 94,000; (3) catalase = 60,000; (4) actin = 43,000; (5) carbonic anhydrase = 29,000; and (6) lysozyme = 14,000.

Following electrophoresis, gels were fixed for 1 h in 50% methanol, 7% glacial acetic acid, then washed by gentle shaking overnight in distilled water. Gels were stained for 20 min in silver stain solution prepared as

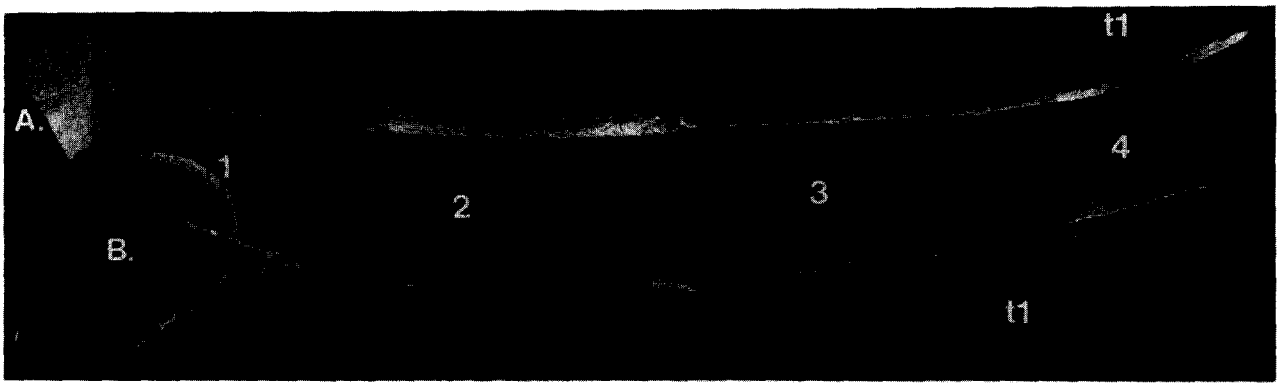

FIGURE 1. Scanning electron micrograph (SEM) collages illustrating the antennae of a *Lygus lineolaris* fifth instar nymph (A) and adult female (B). Note large trichoid sensilla (t1) which are labeled on terminal segments of both nymph and adult. Bar = 500 μm.

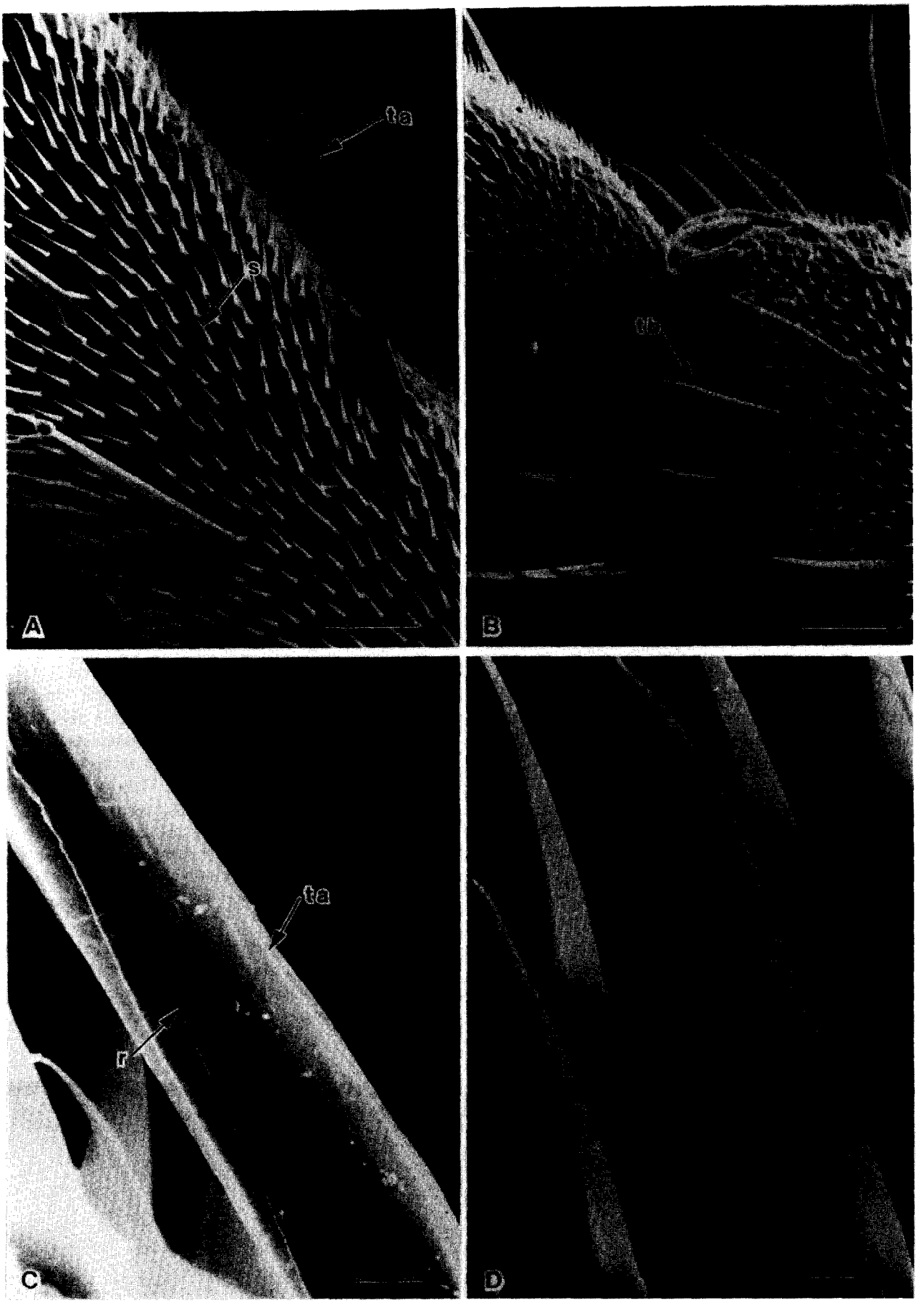

FIGURE 2. Scanning electron micrographs (SEMs) of cuticular structures on second and third antennal segments of *Lygus lineolaris* fifth instar nymphs. (A) Cuticular structures near middle of third antennal segment of a nymph with female reproductive characters. Note large trichoid sensillum (ta) with socketed-base and numerous shorter cuticular structures (s). Bar = 20 μm. (B) Distally-curved trichoid sensilla (tb) with socketed-bases which encircle distal end of second segment. Bar = 20 μm. (C) Higher magnification of midsection of trichoid sensillum (ta) in (A) to show ridged surface structure. Bar = 1 μm. (D) Higher magnification of short cuticular structures (s) in (A). Bar = 1 μm.

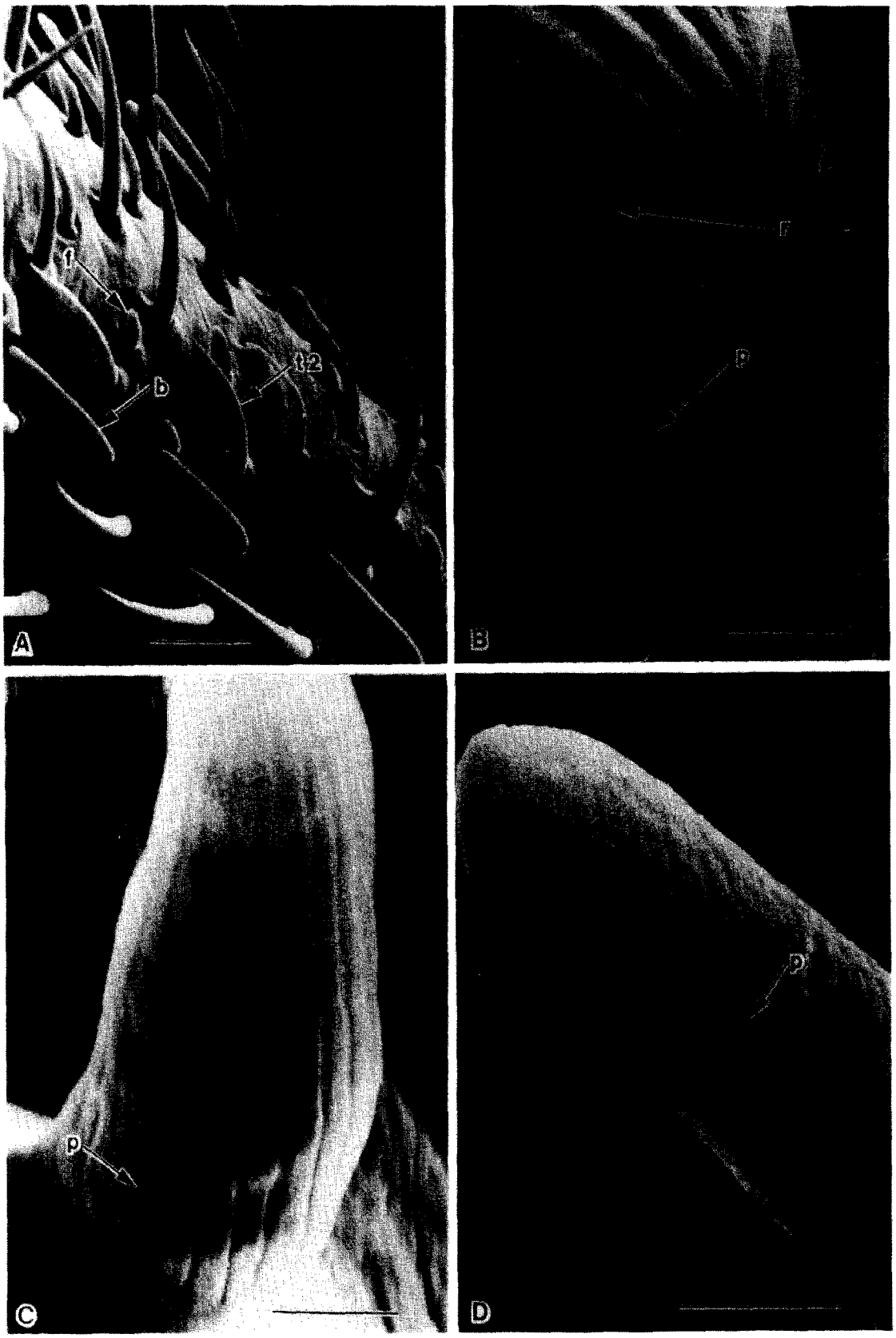

FIGURE 3. Scanning electron micrographs (SEMs) of sensilla on the second antennal segment of an adult *Lygus lineolaris* male. (A) Cuticular structures near middle of second antennal segment: (1) trichoid sensillum (t2) with raised socketed base, diagonal ridges and distally pointed tip; (2) shorter fluted sensillum (f) with somewhat depressed nonsocketed base; (3) long basiconic sensillum (b) with depressed non-socketed base. Bar = 20 μm. (B) Higher magnification of a trichoid sensillum (t2) to show diagonally-ridged (r) surface structure and apparent pit (p), possibly representing a pore. Bar = 1 μm. (C) Higher magnification of fluted sensillum (f) to show longitudinal ridges and pits, possibly representing pores (p). Bar = 1 μm. (D) Higher magnification of tip of basiconic sensillum to show surface pits possibly representing pores (p). Bar = 1 μm.

follows: 0.378 g of NaOH was dissolved in 20 ml of distilled water followed by addition of 7 ml of NH_4OH. $AgNO_3$ (4 g) was then dissolved by vigorous shaking until no brown precipitate remained. The solution was diluted to 500 ml with distilled water and mixed thoroughly. The stained gel was washed for 1 h in distilled water before development in 2% formaldehyde and 0.005% citric acid until the desired intensity was obtained. Development was stopped by resuspension of the gel in 20% methanol, 7% glacial acetic acid.

Protein sequencing

The N-terminal amino acid sequence was determined from protein electroblotted directly on a PVDF membrane using an Applied Biosystems model 475A protein sequencer equipped with a PTH amino acid analyser using the manufactuers protocols. Sequencing was performed by Mr Thomas Fischer, Center for Analysis and Synthesis of Macromolecules, State University of New York, Stony Brook, supported by NIH Grant RR02427 and the Center for Biotechnology.

RESULTS

Morphology

Antennae of *L. lineolaris* nymphs and adults are about 4 mm in total length and consist of four segments (Fig. 1). The largest sensillar type, sensilla trichodea type 1 (t1), is apparent on antennal segments of both nymphs and adults. This sensillar type subtends the antennal surface at an angle of nearly 90°. While numerous sensilla may be discerned on the second, third and fourth segments of the adult antenna shown in Fig. 1(B), large numbers of sensilla are apparent only on the distal segment of the fifth instar nymph antenna shown in Fig. 1(A). No obvious sexual differences were noted in either the size of the antennae or the types of sensilla present either in nymphs with male or female characteristics, or adults. Previous antennal extirpation experiments indicated the second and possibly third segments to be important in the orientation of congeneric adult males to an odorous attractant (Graham, 1988); our morphological results show radical changes in types and numbers of sensilla occur mainly on the second and third segments between nymphs and adults. Therefore, we will limit our descriptions to cuticular structures on these segments and provide a more detailed report of the antennae and associated sensilla elsewhere.

In fifth instar nymphs, cuticular structures on both the second and third antennal segments are similar [Fig. 1(A) and Fig. 2]. Large trichoid sensilla occur along the entire length of both antennal segments [Fig. 1(A) and Fig. 2(A)]. These sensilla have a raised socketed base and longitudinally-ridged surface structure [Fig. 2(C)]. Similar sensilla with raised socketed bases, but which are generally distally-curved, occur in a ring near the distal end of both the second and third segments [Fig. 2(B)]. Numerous shorter spiny cuticular structures occur over the entire length of second and third antennal segments, and the base of the fourth antennal segment [Fig. 1(A) and Fig. 2(A) and (D)]. Surface pits characteristic of pores in the cuticle were not observed in any of these structures.

TABLE 1. Electroantennograms (mean responses in mV ± SE, $n = 3$) of: (1) whole antennae of adult males and nymphs with male sexual characters of *L. lineolaris*; (2) segments 1–3 of adult males, the terminal segment was removed near its junction with the third antennal segment; and (3) segments 1–2 of adult males in which the terminal segment and the penultimate segment were removed near the junction with the second antennal segment

	Odorant	
	1-Hexanol	(*E*)-2-Hexenyl butyrate
Adult (male)		
Whole antenna (1–4)	−0.65 mV (±0.07 mV)	−0.21 mV (±0.11 mV)
Segments 1–3	−0.65 mV (±0.05 mV)	−0.29 mV (±0.05 mV)
Segments 1–2	−0.25 mV (±0.04 mV)	−0.13 mV (±0.02 mV)
Nymph (male)		
Whole antenna (1–4)	−0.03 mV (±0.03 mV)	0 mV

In adult *L. lineolaris*, at least three types of cuticular hairs (in addition to sensilla trichodea type 1 mentioned above) occur on the surface of the second and third antennal segments (Fig. 3). These sensilla have surface pits characteristic of the pores seen in insect olfactory sensilla [Fig. 3(B)–(D)]. A second type of shorter trichoid sensillum (sensillum trichodeum type 2) subtends the surface of the antenna at a lesser angle than sensillum trichodeum type 1, has a raised socketed base, and has a diagonally, strongly-ridged surface structure [Fig. 3(A)–(B)]. Higher magnification micrographs in one instance revealed pits in the walls of this sensillar type. The shortest type of sensilla with pits, the fluted sensilla (f), have longitudinal ridges with pits visible within the grooves [Fig. 3(C)]. A third type of pitted sensillum is a long basiconic (b) type on which pits were observed from its base to its tip [Fig. 3(D)]. These long basiconic sensilla were usually somewhat shorter than the sensilla trichodea type 2.

Electrophysiology

Electroantennograms (EAGs) recorded from whole antennae of adult male *L. lineolaris*, and antennae with the terminal segment removed did not differ in magnitude for either the common plant odorant, 1-hexanol, or the insect-produced volatile, (*E*)-2-hexenyl butyrate (Table 1; Fig. 4). Removal of both the terminal and penultimate segments of the adult antennae resulted in significantly decreased EAGs for both odorants. A small EAG depolarization was recorded from only one of three nymphs [Fig. 4(B)].

Polyacrylamide gel electrophoresis

To determine if sexual differences in antennal proteins of adult *L. lineolaris* occur, the soluble fractions of antennal homogenates of male and female antennae were analyzed by two-dimensional PAGE (Fig. 5). Other than

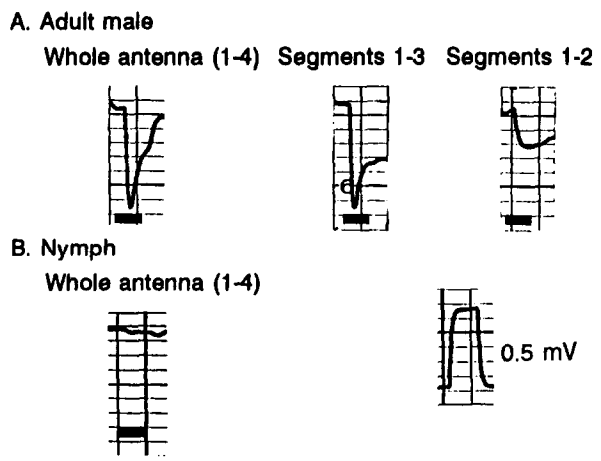

FIGURE 4. Electroantennograms (=EAGs) recorded from *Lygus lineolaris* in response to volatiles emanating from a 50 μg stimulus load of 1-hexanol. (A) EAGs recorded from whole antenna of adult male, segments 1–3 only, and segments 1–2 only. Latter two responses are from contralateral antennae of same individual. (B) EAG recorded from whole antenna of nymph with male abdominal characteristics. Horizontal bar beneath each trace represents duration of stimulus (=1 s).

slight differences in intensities for some polypeptides, no obvious sexual differences in the antennal proteins were detected. The apparent difference between male and female in mobility of a broad band at about 90 kDa and pH = 7 is an anomaly and was not observed in other paired replicate gels.

Considering the obvious differences between nymphs and adults found in our morphological and electrophysiological studies, we looked for related differences in antennal proteins. Native (non-denaturing) and SDS (denaturing) gels were run on soluble proteins of nymphs and adults [Fig. 6(A)]. Both gels show distinct patterns of gene products between these two stages of antennal development. The most striking feature observed was the presence in the native gel of a densely staining band in both adult males and females which was clearly absent from the nymphs [arrow Fig. 6(A)]. A second experiment in which soluble fractions of both prothoracic legs and antennae of nymphs and adults were run on a native gel, showed the polypeptide to be absent in legs thus supporting its localization to adult antennae [Fig. 6(B)]. We routinely observed a slightly greater abundance of this protein in males than in females based on differences in staining intensities.

The polypeptide composition of the adult antennal-specific protein discovered in our previous experiments was determined by: (1) resolving the protein from soluble fractions of male and female antennae on native gels; (2) excising the individual bands; (3) then re-running the excised protein under SDS–PAGE conditions. Results of this experiment revealed what appears to be a single polypeptide with identical molecular weights of 17 kDa for both sexes [Fig. 7(A)]. To determine if our 17 kDa antennal proteins were related to previously identified odorant-binding proteins of similar molecular weight in moths, cross-reactivity was tested on immunoblots using antisera obtained against pheromone-binding protein in the gypsy moth, *Lymantria dispar* (Vogt *et al.*, 1989). While the antisera cross-reacting with a protein from the soluble fraction of antennae of the male beet armyworm moth, *Spodoptera exigua*, served as a positive control, no reaction was apparent with antennal proteins of either sex of *L. lineolaris* [Fig. 7(B)].

Protein sequence

The adult antennal-specific protein was separated for sequencing from other proteins occurring in the soluble antennal fraction using non-SDS–PAGE. Conveniently

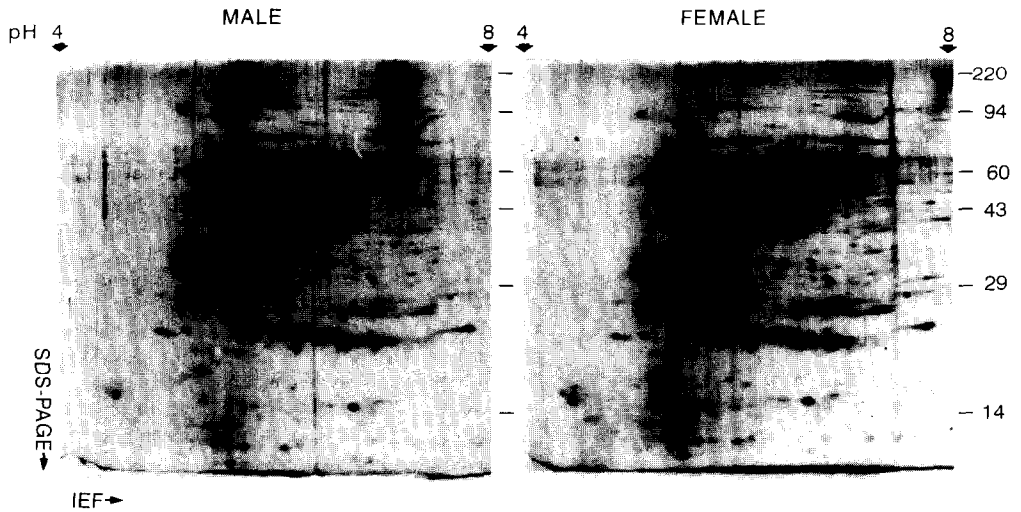

FIGURE 5. Two-dimensional PAGE of soluble fractions of antennal homogenates of adult *Lygus lineolaris* males and females. The pH range of the first dimension gel (isoelectric focusing = IEF) is indicated above the gels. The positions of protein standards (kDa) used in the second dimension gel (SDS–PAGE) are indicated at the right of the gels. The arrows locate tropomyosin, a 32.7 kDa (pI = 5.2) protein, which served as an internal standard. The apparent difference between male and female in mobility of a broad band at about 90 kDa and pH = 7 is an anomaly and was not observed in other paired replicate gels.

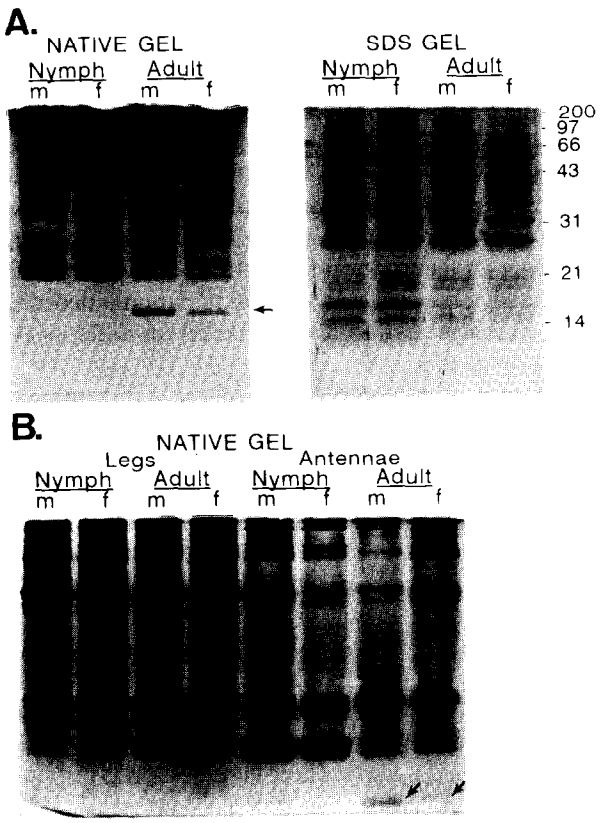

FIGURE 6. (A) One-dimensional native (non-denaturing) and SDS (denaturing) gels of soluble fractions of antennal homogenates of nymphs with male and female characteristics, and adult male and female *L. lineolaris*. Arrow indicates polypeptide present in native gel of adult males and females which is absent in nymphs. The positions of protein standards (kDa) used in the SDS gel are indicated on the right. (B) Native (non-denaturing) gel of soluble fractions of prothoracic legs and antennae of nymphs with male and female characteristics, and adult male and female *L. lineolaris*. Arrow indicates polypeptide present in native gel of adult male and female antennae which is absent in legs of adults, and legs and antennae of nymphs.

under non-SDS–PAGE conditions, the desired protein migrated to the bottom of the gel, well-resolved from other proteins. Proteins resolved on the non-SDS gel were electroblotted onto a PVDF membrane. The antennal-specific protein was visualized following brief Coomassie staining. This protein band excised from the blot was used for microsequencing of the N-terminal. The N-terminal sequence obtained for the adult antennal-specific protein was:

G E L P E E M R E M A Q G L H D X G V E

DISCUSSION

Morphology

Types, numbers and distribution of antennal sensilla of *L. lineolaris* change dramatically from fifth instar nymph to adult (final molt). These changes are most apparent on the second and third segments in which nymphs have a scattered population of large trichoid sensilla with a ridged surface structure, similar sensilla which are curved distally ringing the distal end of both segments and numerous cuticular spines over much of the surface of both segments. By comparison, these two segments in adults have similar large trichoid sensilla scattered over the surface of both segments, and distally-curved trichoid sensilla ringing the terminal end

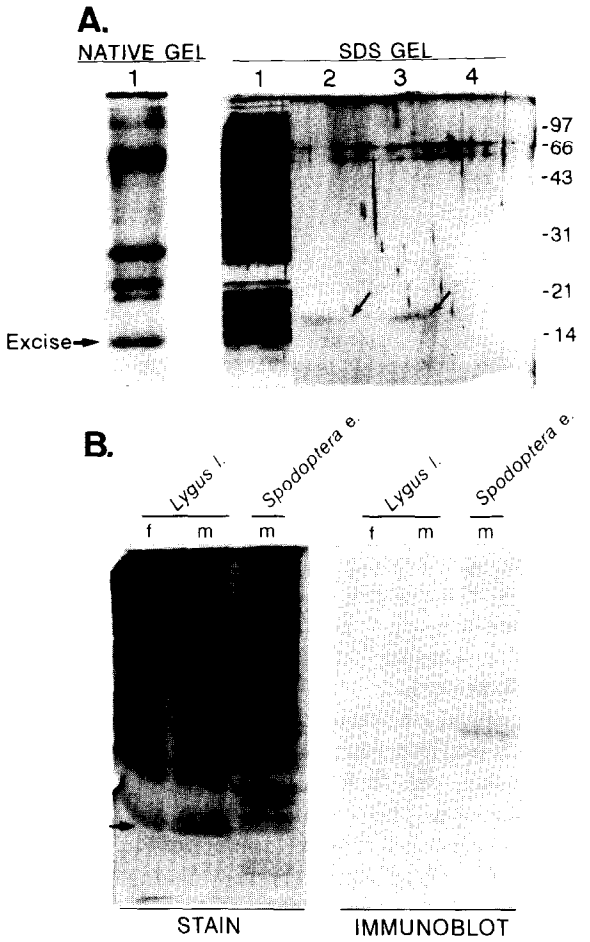

FIGURE 7. Polypeptide composition of adult antennal-specific protein of adult *L. lineolaris*. (A) Soluble protein fraction from antennal homogenate of adult male insects was run on a native gel in order to resolve the adult-specific protein (lane 1, native gel). Following brief staining of the gel, the protein band was excised (arrow, native gel) and incubated for 45 min in 100 μl of 3 × SDS sample buffer (Materials and Methods). The gel slice was then loaded on an SDS gel and electrophoresed under denaturing conditions revealing a single polypeptide (arrow, lane 2). Lane 3 is as described for lane 2 except the native gel band was from the soluble fraction of adult female antennae. Lane 4 is a control using a gel slice from a blank lane of the native gel. Lane 1 of the SDS gel shows the original soluble fraction of the male antennae run under denaturing conditions. The positions of protein standards (kDa) are shown on the right of the SDS gel. (B) Soluble protein fractions from antennal homogenates of male (m) and female (f) *L. lineolaris* and male (m) *Spodoptera exigua* were resolved on a native gel then electroblotted onto nitrocellulose. Shown on the left is a portion of the same native gel which was Coomassie stained (STAIN) instead of blotted. The arrow indicates the position of the adult antennal-specific protein in the male and female *L. lineolaris* samples. The blotted portion of the native gel (IMMUNOBLOT) was incubated with antisera raised against the pheromone binding protein of *Lymantria dispar* as described in Materials and Methods . The antisera recognized a single protein in the *S. exigua* sample but did not react with the *L. lineolaris* antennal proteins.

of both segments. None of the aforementioned sensilla in nymphs or adults have apparent multiple wall pores characteristic of olfactory chemosensilla (Altner, 1977; Zacharuk, 1980; Dickens and Payne, 1986) based on scanning electron microscopy. Additionally on the second and third segments of adults of both sexes, three other types of sensilla occur with surface pits probably representing pores. The most numerous porous sensillum types, the diagonally-ridged sensilla trichodea type 2, had apparent pores between the ridges, while the long sensilla basiconica have pores from their base to the tip. The less numerous fluted sensilla have a grooved surface structure in which pores are apparent in the grooves.

Various aspects of development of antennae and associated sensilla have been reported for several insects, principally endopterygote species of Lepidoptera, Coleoptera and Diptera, and exopterygote Orthoptera and Heteroptera (Zacharuk and Shields, 1991). Most of these studies have dealt with either changes in numbers and types of sensilla during development, or cellular morphogenesis. For hemimetabolous insects (exopterygotes), such as the locust, *Locusta migratoria* (Orthoptera: Acrididae), the greatest increase in numbers of olfactory sensilla occurs at the final molt (Chapman and Greenwood, 1986). In cockroaches of the genus *Periplaneta*, numbers of olfactory chemosensilla increase at each molt for both sexes, but following the final molt, males have nearly twice as many olfactory sensilla as females (Schafer and Sanchez, 1976b). Changes in the numbers of olfactory sensilla with the onset of adulthood were thought to be associated with the reception of an airborne pheromone for initiating courtship. Zacharuk and Shields (1991) point out that changes in sensillar numbers at the final molt for the endopterygotes correlates with sexual function and changes in patterns of behavior.

Sexual dimorphism and changes in number and distribution of sensilla at the final molt are variable for Heteroptera examined to date. For example, no sexual differences in the antennae and associated sensilla are apparent for either the small milkweed bug, *Lygaeus kalmii* (Hemiptera: Lygaeidae) (Slifer and Sekhon, 1963) or the large milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae) (Harbach and Larsen, 1976). Neither sexual differences nor differences in antennae of nymphs and adults were observed for the bedbug, *Cimex lectularius* (Levinson *et al.*, 1974; Steinbrecht and Müller, 1976). In contrast, antennae of *Rhodnius pictipes* nymphs differ from antennae of adults in the absence of basiconic and trichoid sensilla on the first flagellar segment and proximal half of the second flagellar segment of the former (Catalá and Schofield, 1994). No sexual differences were apparent among the ten *Rhodnius* species examined. Similarly, while no sexual differences were observed in adult antennae of *Riptortus clavatus* (Heteroptera: Alydidae), antennae of nymphs differed from adults in number and distribution of sensilla and cuticular denticles (Ikeda-Kikue and Numata, 1991).

The development of putative olfactory sensilla on the second and third antennal segments of adult *L. lineolaris* are consistent with the acquisition of sexual behavior. Virgin females of *L. lineolaris* attract males both in field (Scales, 1968; Graham, 1987) and in laboratory (Graham, 1987, 1988) behavioral studies. Antennal extirpation experiments in the closely related species, *L. hesperus*, showed that removal of either the terminal antennal segment or the distal two segments in males resulted in no decrease in the percent of males responding to female volatiles in laboratory bioassays (Graham, 1988).

Electrophysiology

Electrical responses recorded from the antennal preparations correlate with the development of presumably olfactory sensilla on the second and third antennal segments during the molt from the fifth instar nymph to the adult. The fact that EAGs did not decrease with excision of the terminal antennal segment indicates that receptor neurons responsive to the insect-produced volatile and plant odor occur principally on the second and third antennal segments. These results correlate with behavioral studies in which removal of the terminal antennal segment of males had no significant effect on their response to a female attractant in a laboratory olfactometer (Graham, 1988). Extirpation of the terminal and penultimate segments did not result in decreased behavioral responses of males to the female attractant in the lab. The fact that significant though reduced EAGs could be recorded from the first and second antennal segments of males alone, indicates some receptors for the female attractant must occur on the second segment.

Our results may be compared with those obtained for another hemimetabolous insect, the cockroach, *Periplaneta americana* (Schafer, 1977b). As mentioned previously, numbers of olfactory sensilla increase significantly in male *P. americana* compared to females at the final molt (Schafer and Sanchez, 1976b). While EAGs to several odorants may be recorded from larvae (nymphs) and females, significantly larger EAGs are recorded from males, especially for a female-produced attractant. Numbers of olfactory sensilla, magnitude of EAGs in response to a female-produced attractant and behavioral responses of males to the female-produced attractant were all decreased by topical application of a juvenile hormone mimic (Schafer and Sanchez, 1976a; Schafer, 1977a,b). In the bedbug, *Cimex lectularius*, behavioral responses to an alarm pheromone could be correlated with electrophysiological responses of sensilla on the terminal antennal segment (Levinson *et al.*, 1974). Diminished behavioral responses of bedbugs to the alarm pheromone following extirpation of the terminal segment was related to the lack of olfactory sensilla on the other antennal segments (Levinson *et al.*, 1974; Steinbrecht and Müller, 1976).

Antennal-specific protein

This is the first report of an antennal-specific protein in a hemimetabolous insect. Previously reported antennal-

specific proteins include: (1) pheromone-binding proteins (PBP) (Vogt and Riddiford, 1981; Klein, 1987; Gyorgyi *et al.*, 1988; Raming *et al.*, 1989, 1990; Vogt *et al.*, 1991; Krieger *et al.*, 1993) and general odorant binding proteins (GOBP) (Breer *et al.*, 1990; Vogt *et al.*, 1991; Krieger *et al.*, 1993) from several species of adult Lepidoptera, and the dipteran, *Drosophila melanogaster* (McKenna *et al.*, 1994) that have molecular weights of around 16,000 (Vogt *et al.*, 1993) and are abundant in soluble fractions of whole antennae and sensilla; (2) antennal-specific esterases in moths that have a molecular mass of between 55,000 and 90,000 (Vogt and Riddiford, 1981; Vogt *et al.*, 1985; Vogt, 1987); and (3) a pheromone selective binding protein, ApolSHMP69, associated with dendritic membranes of trichoid sensilla of male *Antheraea polyphemus* (Vogt *et al.*, 1988) which has a molecular weight of 69,000. Among antennal-specific proteins found in other insects, both the relatively great abundance and molecular weight of our antennal-specific protein are similar to pheromone binding proteins of moths and *Drosophila*.

The fact that our antennal-specific protein is differentially expressed in adult *L. lineolaris* compared with nymphs correlates with the development of wall-pore sensilla on the second and third antennal segments. Single sensillum recordings from sensilla on these segments reveal neurons with spontaneous activity which respond to stimulation by odors such as the insect-produced compounds, hexyl butyrate and (*E*)-2-hexenyl butyrate, all of which are characteristic of olfactory receptor neurons (Dickens, unpublished). Our results shown here suggest that male antennae appear to have a higher concentration of the antennal-specific protein than female antennae; this correlates with the greater number of sensilla found on male antennae (Chinta and Dickens, unpublished), and is consistent with the larger electroantennograms recorded from male antennae to such insect-produced odorants as (*E*)-2-hexenyl butyrate and hexyl butyrate (Chinta *et al.*, 1994).

Differences occur in hormonal levels during progression of the final instars to adults between hemimetabolous and holometabolous insects (Riddiford, 1994). Expression of odorant binding proteins in holometabolous (endopterygote) moths is apparently induced by a decline in ecdysteroids in the pupal stage prior to ecdysis (Vogt *et al.*, 1993). In hemimetabolous (exopterygote) cockroaches, topical application of a juvenile hormone mimic to final instar nymphs of *P. americana*, leads to reduction of olfactory sensilla, probably containing receptors for sex attractants, in males (Schafer and Sanchez, 1976a), while injection of juvenile hormone into final instar nymphs in *Blatella germanica* reduces all types of sensilla in adult females (Ramaswamy and Gupta, 1981). The adult antennal-specific protein we describe here for *L. lineolaris* affords us an opportunity to examine endocrine regulation of antennal or olfactory development in a hemimetabolous insect in which development from final instar to adult is less protracted than in holometabolous insects.

The N-terminal sequence for twenty amino acids obtained for our adult antennal-specific protein was searched on the National Center for Biotechnology Information BLAST e-mail server, and was found to have no significant homology to other insect proteins. If this antennal-specific protein functions as an odorant-binding protein as we suspect, it is either widely diverged or independently derived.

In summary, we have shown that sensilla with surface pits probably representing pores and housing olfactory receptor neurons develop on the second and third antennal segments during the final molt in *L. lineolaris*. Corresponding with the development of porous sensilla on these segments, neural responses to both a common plant volatile and an insect-produced odorant increase. The expression of an antennal-specific protein with a molecular weight of about 17,000 in adults correlates with both the morphological changes and results of our electrophysiological studies. The localization of this protein in the antennae and its molecular weight correspond favorably with the odorant-binding proteins found in several moth species and *Drosophila*. The fact that the antennal-specific protein has no significant homology with other insect proteins, including odorant binding proteins, indicates that if it functions as an OBP, it is either widely divergent or independently derived.

REFERENCES

Aldrich J. R., Lusby W. R., Kochansky J. P., Hoffman M. P., Wilson L. T. and Zalom F. G. (1988) *Lygus* bug pheromones vis-à-vis stink bugs. *Proc. Beltwide Cotton Conf.*, pp. 213–216.

Altner H. (1977) Insect specificity and structure: an approach to a new typology. In *Olfaction and Taste VI* (Eds LeMagnen J. and MacLeod P.), pp. 295–303. Information Retrieval Ltd, Washington, DC.

Breer H., Krieger J. and Raming K. (1990) A novel class of binding proteins in the antennae of the silk moth *Antheraea pernyi*. *Insect Biochem.* **20,** 735–740.

Catalá S. and Schofield C. (1994) Antennal sensilla of *Rhodnius*. *J. Morphol.* **219,** 193–203.

Chapman R. F. and Greenwood M. (1986) Changes in abundance of antennal sensilla during growth of *Locusta migratoria* L. (Orthoptera: Acrididae). *Int. J. Insect Morphol. Embryol.* **15,** 83–96.

Chinta S., Dickens J. C. and Aldrich J. R. (1994) Olfactory reception of potential pheromones and plant odors by tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae). *J. Chem. Ecol.* **20,** 3251–3267.

Dickens J. C., Visser J. H. and Van Der Pers J. N. C. (1993) Detection and deactivation of pheromone and plant odor components by the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *J. Insect Physiol.* **39,** 503–516.

Dickens J. C. and Payne T. L. (1986) Chemical messengers and insect behavior. In *Handbook of Natural Pesticides: Methods Volume I Theory, Practice, and Detection* (Ed. Mandava N. B.), pp. 201–230. CRC Press, Boca Raton, FL.

Feir D. and Beck S. D. (1963) Feeding behavior of the large milkweed bug, *Oncopeltus fasciatus*. *Ann. Ent. Soc. Am.* **56,** 224–229.

Graham H. M. (1987) Attraction of *Lygus* spp. males by conspecific and congeneric females. *Southwest. Ent.* **12,** 147–155.

Graham H. M. (1988) Sexual attraction of *Lygus hesperus* Knight. *Southwest. Ent.* **13,** 31–37.

Gueldner R. C. and Parrott W. L. (1978) Volatile constituents of the tarnished plant bug. *Insect Biochem.* **8,** 389–391.

Gyorgyi T. K., Roby-Shemkovitz A. J. and Lerner M. R. (1988) Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: a tissue-specific developmentally regulated protein. *Proc. natn. Acad. Sci. U.S.A.* **85,** 9851–9855.

Harbach R. E. and Larsen J. R. (1976) Ultrastructure of sensilla on the distal antennal segment of adult *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). *Int. J. Insect Morphol. Embryol.* **5,** 23–33.

Ikeda-Kikue K. and Numata H. (1991) Compensatory regeneration of antennae after removal of the distal segment in *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae). *Int. J. Insect Morphol. Embryol.* **20,** 41–49.

Kaissling K.-E. (1974) Sensory transduction in insect olfactory receptors. In *Biochemistry of Sensory Functions* (Ed. Jaenicke L.). pp. 243–273. Springer, Berlin.

Kaissling K.-E. (1986) Chemo-electrical transduction in insect olfactory receptors. *A. Rev. Neurosci.* **9,** 121–145.

Klein U. (1987) Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem.* **17,** 1193–1204.

Krieger J., Gänßle H., Raming K. and Breer H. (1993) Odorant binding proteins of *Heliothis virescens*. *Insect Biochem. Molec. Biol.* **23,** 449–456.

Laemmli U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Laue M., Steinbrecht R. A. and Ziegelberger G. (1994) Immunocytochemical localization of general odorant-binding protein in olfactory sensilla of the silkmoth *Antheraea polyphemus*. *Naturwissenschaften* **81,** 178-180.

Levinson H. Z., Levinson A. R., Müller B. and Steinbrecht R. A. (1974) Structure of sensilla, olfactory perception, and behaviour of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. *J. Insect Physiol.* **20,** 1231–1248.

Marder J. B., Mattoo A. K. and Edelman M. (1986) Identification and characterization of the psbA gene product: the 32-kDa chloroplast membrane protein. *Meth. Enzymol.* **118,** 384–396.

McKenna M. P., Hekmat-Scafe D. S., Gaines P. and Carlson J. R. (1994) Putative *Drosophila* pheromone-binding proteins expressed in a subregion of the olfactory system. *J. biol. Chem.* **269,** 16340–16347.

O'Farrell P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. biol. Chem.* **250,** 4007–4021.

Pantle C. and Feir D. (1976) Olfactory responses to milkweed seed extracts in the milkweed bug. *J. Insect Physiol.* **22,** 285–289.

Ramaswamy S. B. and Gupta A. P. (1981) Sensilla of the antennae and the labial and maxillary palps of *Blatella germanica* (L.) (Dictyoptera: Blatellidae): their classification and distribution. *J. Morphol.* **168,** 269–279.

Raming K., Krieger J. and Breer H. (1989) Molecular cloning of an insect pheromone-binding protein. *FEBS Lett.* **256,** 215–218.

Raming K., Krieger J. and Breer H. (1990) Primary structure of a pheromone-binding protein from *Antheraea pernyi*: homologies with other ligand-carrying proteins. *J. comp. Physiol. B* **160,** 503–509.

Riddiford L. M. (1994) Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. *Adv. Insect Physiol.* **24,** 213–274.

Scales A. L. (1968) Female tarnished plant bugs attract males. *J. Econ. Ent.* **61,** 1466–1467.

Schafer R. (1977a) The nature and development of sex attractant specificity in cockroaches of the genus *Periplaneta* III. Normal intra-and interspecific behavioral responses and responses of insects with juvenile hormone-altered antennae. *J. exp. Zool.* **199,** 73–84.

Schafer R. (1977b) The nature and development of sex attractant specificity in cockroaches of the genus *Periplaneta* IV. Electrophysiological study of attractant specificity and its determination by juvenile hormone. *J. exp. Zool.* **199,** 189–207.

Schafer R. and Sanchez T. V. (1976a) The nature and development of sex attractant specificity in cockroaches of the genus *Periplaneta* II Juvenile hormone regulates sexual dimorphism in the distribution of antennal olfactory receptors. *J. exp. Zool.* **198,** 323–336.

Schafer R. and Sanchez T. V. (1976b) The nature and development of sex attractant specificity in cockroaches of the genus *Periplaneta* I Sexual dimorphism in the distribution of antennal sense organs in five species. *J. Morphol.* **149,** 139–158.

Schneider D. (1957) Elektrophysiologische Untersuchungen von Chemo-und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori*. *Z. vergl. Physiol.* **40,** 8–41.

Slifer E. and Sekhon S. S. (1963) Sense organs on the antennal flagellum of the small milkweed bug, *Lygaeus kalmii* Stal (Hemiptera, Lygaeidae). *J. Morphol.* **112,** 165–193.

Snodgrass G. L. and McWilliams J. M. (1992) Rearing of the tarnished plant bug (Heteroptera: Miridae) using a tissue paper oviposition site. *J. Econ. Ent.* **85,** 1162–1166.

Snodgrass G. L., Scott W. P. and Smith J. W. (1984) Host plants and seasonal distribution of the tarnished plant bug (Hemiptera: Miridae) in the Delta of Arkansas, Louisiana, and Mississippi. *Envir. Ent.* **13,** 110–116.

Steinbrecht R. A. and Müller B. (1976) Fine structure of the antennal receptors of the bedbug, *Cimex lectularius* L. *Tissue Cell* **4,** 615–636.

Steinbrecht R. A., Ozaki M. and Ziegelberger G. (1992) Immunocytochemical localization of pheromone-binding protein in moth antennae. *Cell Tissue Res.* **270,** 287-302.

Visser J. H. (1986) Host odor perception in phytophagous insects. *A. Rev. Ent.* **31,** 121–144.

Vogt R. G. (1987) The molecular basis of pheromone reception: its influence on behavior. In *Pheromone Biochemistry* (Eds Prestwich G. D. and Blomquist G. J.), pp. 385–431. Academic Press, New York.

Vogt R. G. and Riddiford L. M. (1981) Pheromone binding and inactivation in moth antennae. *Nature* **293,** 161–163.

Vogt R. G. and Riddiford L. M. (1986) Pheromone reception: a kinetic equilibrium. In *Mechanisms in Insect Olfaction* (Eds Payne T. L., Birch M. L. and Kennedy C. E. J.), pp. 201–208. Oxford University Press, Oxford.

Vogt R. G., Riddiford L. M. and Prestwich G. D. (1985) Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. natn. Acad. Sci. U.S.A.* **82,** 8827–8831.

Vogt R. G., Prestwich G. D. and Riddiford L. M. (1988) Sex pheromone receptor proteins. Visualization using radiolabeled photoaffinity analog. *J. biol. Chem.* **263,** 3952–3959.

Vogt R. G., Köhne A. C., Dubnau J. T. and Prestwich G. D. (1989) Expression of pheromone binding proteins during antennal development in the Gypsy moth *Lymantria dispar*. *J. Neurosci.* **9,** 3332–3346.

Vogt R. G., Prestwich G. D. and Lerner M. R. (1991) Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol.* **22,** 74–84.

Vogt R. G., Rybczynski R., Cruz M. and Lerner M. R. (1993) Ecdysteroid regulation of olfactory protein expression in the developing antenna of the tobacco hawk moth, *Manduca sexta*. *J. Neurobiol.* **24,** 581–597.

Zacharuk R. (1980) Ultrastructure and function of insect chemosensilla. *A. Rev. Ent.* **25,** 27–47.

Zacharuk R. and Shields V. (1991) Sensilla of immature insects. *A. Rev. Ent.* **36,** 331–354.

Acknowledgements—We are especially grateful to Professor R. G. Vogt, Department of Biological Sciences, University of South Carolina, Columbia, for both advice and encouragement, and providing antisera raised against pheromone binding protein of *Lymantria dispar*; and Dr G. L. Snodgrass, USDA, ARS, Southern Insect Management Laboratory, Stoneville, MS, for graciously providing insects without which this study would not have been possible. Both Professor Vogt and Dr A. Mattoo, USDA, ARS, Plant Molecular Biology Laboratory, Beltsville, MD were helpful in searching databases for sequence homologies. We thank Professor R. G. Vogt; Professor R. J. O'Connell, Worcester Foundation for Experimental Biology, Worcester, MA; Professor A. R. Alford, Department of Environmental Biology and Applied Ecology, University of Maine, Orono; Professor F. E. Hanson, Department of Biological Sciences, University of Maryland, Baltimore; and Dr M. S. Mayer, USDA, ARS, Insect Attractants, Behavior and Basic Biology Laboratory, Gainesville, FL for helpful comments on the manuscript.